Exhibit E3

MHC Class I-Dependent Presentation of Exoerythrocytic Antigens to CD8$^+$ T Lymphocytes Is Required for Protective Immunity Against *Plasmodium berghei*[1,2]

Katherine L. White,*† Heidi Link Snyder,[3]*† and Urszula Krzych[4]*†

T lymphocytes are believed to play a major role in protection against malaria. Previous experiments using in vivo depletion of CD8$^+$ T cells, reconstitution with CD8$^+$ T splenic cells, and adoptive transfer of CD8$^+$ CTL clones demonstrated that protection against the exoerythrocytic stage of the murine strain, *Plasmodium berghei* malaria, was CD8$^+$ T cell-dependent. Despite evidence for the critical role of CD8$^+$ CTL, neither the cellular nor the molecular requirements for CD8$^+$ T cell induction or for recognition of malaria Ags are known. In this study, we wished to define the role of CD8$^+$ T cells and MHC class I molecules by using the *P. berghei* malaria attenuated sporozoites (SPZ) protection model in β_2-microglobulin (β_2m) knockout (−/−) mice. In contrast to observations that $\beta_2m^{-/-}$ mice are resistant to many infectious diseases by compensatory mechanisms involving non-class I-restricted T cells, we found that $\beta_2m^{-/-}$ mice failed to be protected against *P. berghei* SPZ, although immunization with attenuated SPZ induced production of IL-2, INF-γ, anti-circumsporozoite protein IgG, and proliferative T cells. The lack of compensatory mechanisms involving non-CD8$^+$ T cells was particularly evident in the failure to adoptively transfer protective immunity with wild-type SPZ-immune splenic T cells. From our data it can be concluded that CD8$^+$ T cells induced during immunization with attenuated SPZ must recognize liver-expressed Ags presented by class I molecules to engage effectively in a response leading to destruction of the malaria parasites. *The Journal of Immunology,* **1996, 156: 3374–3381.**

Malaria is a tropical infectious disease that claims millions of lives annually. Natural exposure to malaria infection through a bite from a plasmodia-infected *Anopheles* mosquito does not generally result in long-lasting, sterile protection, partly because malaria Ags are poorly immunogenic (1, 2). In contrast, repeated exposure to bites from radiation-attenuated plasmodia-infected mosquitoes induces protection against an infectious challenge in humans (3) and rodents (4), and both models have been used as a framework to investigate immune responses that might confer protection.

Although attenuated sporozoites (SPZ)[5] fail to establish an erythrocytic-stage infection, they are able to invade the liver, carrying with them the SPZ-stage-associated Ags, circumsporozoite protein (CSP), and SPZ surface protein-2 (SSP2) (5). Irradiated parasites also express liver- and blood-stage Ags (6). The persistence of attenuated *Plasmodium berghei* SPZ in the liver of SPZ-immune rodents is critical for the induction and maintenance of protective immunity (7). T cells responding to Ags associated with liver- and blood-stage infections have been detected in PBL of attenuated *Plasmodium falciparum* SPZ-immune volunteers, suggesting that these specificities might have been induced by liver-accumulated Ags (8).

On the basis of observations from the attenuated-SPZ model, it is believed that cytolytic T lymphocytes (CTL) are the prominent anti-pre-erythrocytic stage effector cells; however, an integrated multifactorial immune mechanism may also participate in protective immunity against malaria (9–13). CD8$^+$ CTL specific for class I MHC-associated peptides from CSP and SSP2 have been found in both murine (14–17) and human (18–19) lymphocytes exposed to irradiated SPZ and liver-stage Ag peptide-specific CTL have been shown in persons living in areas of malaria hyperendemicity (20). The involvement of CD8$^+$ T cells has been corroborated in studies showing that systemic depletion of CD8$^+$ T cells (21, 22) interferes with protective immunity and, conversely, that adoptive transfer of CD8$^+$ T cell clones specific for CSP (23, 24) prevents parasitemia induced by infectious SPZ. Furthermore, reduction of the parasite load has been noted in livers of protected animals compared with nonimmune controls (15). Final conclusions regarding the mechanisms of T cell induction and recognition by liver-expressed Ags have not been reached because the requirements for activation of protective lymphocytes remain unknown.

We adopted the β_2-microglobulin (β_2m) knockout ($\beta_2m^{-/-}$) mouse strain (25) to investigate whether mechanisms independent of MHC class I-restricted CD8$^+$ CTL might mediate antimalaria protection induced by attenuated *P. berghei* SPZ. Having disrupted the β_2m gene by homologous recombination, $\beta_2m^{-/-}$ mice do not express MHC class I molecules and therefore fail to export class I-associated peptides for induction, activation, or recruitment of CD8$^+$ T cells (25). $\beta_2m^{-/-}$ mice have been used to study

*Department of Biology, The Catholic University of America, Washington, DC, 20064; and †Department of Immunology, Walter Reed Army Institute of Research, Washington, DC, 20307

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[3] Current address: Department of Viral Diseases, NIAID, National Institutes of Health, Bethesda, MD 20892.

[4] Address correspondence and reprint requests to Dr. Urszula Krzych, Department of Immunology, CD&I, Building 40, Walter Reed Army Institute of Research, Washington, DC, 20307-5100.

[5] Abbreviations used in this paper: SPZ, sporozoites; $\beta_2m^{-/-}$ mice, β_2-microglobulin knockout mice; CSP, circumsporozoite protein; SSP2, sporozoite surface protein-2; PBS-TW20, phosphate-buffered 0.025% Tween-20 and 1% Thimerosal.

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autoimmunity (26), tumor immunity (27), and viral (28–30) and protozoan (31, 32) infections. In these systems, $CD8^+$ T cells are either associated with the development of disease or are believed to mediate key immunologic functions. Most notably, in studies in which $CD8^+$ T cells have been thought to be the primary effectors responsible for clearance of vaccinia, influenza, and lymphocytic choriomeningitis virus, the viral diseases were prevented by the induction of $CD4^+$ MHC class II-restricted CTL in the $\beta_2m^{-/-}$ mice (28–30). Compensatory effects involving NK cells were also observed in *Toxoplasma gondii*-infected $\beta_2m^{-/-}$ mice (31), suggesting that although $CD8^+$ T cells might play a key role, their reactivity is aided or even replaced by other cell types. In contrast, the absence of $CD8^+$ T lymphocytes during *Trypanosoma cruzi* infection led to high parasitemia that resulted in early death (32).

We have previously established that immunization of C57BL/6 ($\beta_2m^{+/+}$) mice with attenuated *P. berghei* SPZ induces Ag-specific proliferative T cells, anti-CSP-IgG, lymphokines, and, most importantly, protection (33, 34). In the present study, using the attenuated *P. berghei* SPZ protection model, we characterized the immune responses of $\beta_2m^{-/-}$ mice to establish whether the absence of MHC class I molecules affects the anti-SPZ responses, including the induction of protective immunity. We observed that although some SPZ-specific immune reactivities from $\beta_2m^{-/-}$ mice, $\beta_2m^{+/+}$, and $\beta_2m^{+/-}$ mice were comparable, protective immunity was not. From these data we concluded that protection is dependent on effector mechanisms, presumably involving $CD8^+$ T cells, that require the recognition of or interaction with MHC class I molecules within the infected livers.

Materials and Methods

Mice

Female C57BL/6 ($\beta_2m^{+/+}$) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). $\beta_2m^{-/-}$ and $\beta_2m^{+/-}$ (C57BL/6 × 129) mice, homozygous and heterozygous, respectively, for the disrupted β_2m gene as described by Koller (25) were kindly provided by Dr. A. Barnes (National Institutes of Health, Bethesda, MD). All mice were housed in sterile barrier rooms and used at 8 to 12 wk of age.

Culture media

RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Grand Island, NY), 100 U/ml penicillin-streptomycin (Life Technologies), 8 mM L-glutamine (Life Technologies), 0.1 mM nonessential amino acids (Life Technologies), 1 mM sodium pyruvate (Life Technologies), and 100 mM 2-ME (Bio-Rad, Richmond, CA) was the culture medium. HBSS (Life Technologies) was used without supplement. PBS (Life Technologies) was used alone or supplemented with 0.025% Tween-20 (Bio-Rad) and 1% Thimerosal (Bio-Rad) and designated PBS-TW20.

Sporozoite preparation

Sporozoites (*P. berghei* NK65 strain) dissected from *Anopheles stephensi* mosquitoes as previously described (33) were provided by Dr. Imogene Schneider (Department of Entomology, Walter Reed Army Institute of Research). Sporozoites were attenuated by exposure to 15,000 rad irradiation with a Gamma Cell 220, cobalt 60 irradiator (Atomic Energy, Canada) and kept on ice until used. Sporozoites intended for use in culture were irradiated at 30,000 rad as a precautionary measure to eliminate contaminants that may have arisen after nonsterile dissection of the mosquitoes, aliquoted, and stored at −70°C until the time of assay. Sporozoites intended for infectivity studies were used immediately after dissection without further treatment.

Immunizations

All mice were immunized i.v. in the tail vein with either a single dose of 75,000 irradiated SPZ administered in 0.2 ml of medium 199 (Life Technologies) or a single dose of 75,000 irradiated SPZ followed by two weekly boosts with 20,000 (75,000, 20,000, 20,000) irradiated SPZ. Hyperimmunized mice received 100,000 irradiated SPZ followed by three 20,000 boost immunizations with irradiated SPZ (100,000, 20,000, 20,000, 20,000) given 1 wk apart.

Protection and parasitemia screening

Parasitemia was determined as described (33, 34). Briefly, more than 40 mice from each SPZ-immunized group and naive animals were challenged i.v. with 10,000 or 1,000 infectious *P. berghei* (NK65 strain) SPZ and the presence of parasitemia was screened by thin blood smears on individual animals starting at day 2 postchallenge. Two hundred erythrocytes were screened microscopically for the presence of parasites and mice were assumed protected if no parasitemia was detected by day 14 postchallenge.

Adoptive transfer experiments

The entire splenic population from SPZ-immune mice was prepared into a single-cell suspension and adjusted to 1×10^8 cells/0.2 ml in PBS and transferred into mice via the tail vein. Transfers of immune splenic cells were performed on day 5 following the final 20,000 irradiated SPZ immunization. Two days after splenic transfer, animals were challenged with 10,000 infectious *P. berghei* SPZ and protection was determined as described above.

Cell preparation

One week after the final immunization, mice were killed according to the *Guide for the Care and Use of Laboratory Animals* from the Institute for Laboratory Resources, National Research Council, and the spleens were removed aseptically and placed in HBSS. Single-cell lymphocyte suspensions (33, 34) were washed three times by centrifugation (250 × *g* for 10 min) at room temperature. After the final wash, cell densities were adjusted to 5×10^6 cells/ml in culture medium.

Proliferation assays

Proliferation assays were performed as described (34). Briefly, splenic lymphocytes were cultured in 96-well microtiter plates (Costar, Cambridge, MA) at a final concentration of 5×10^5 cells/0.2 ml of culture medium containing irradiated SPZ at 100, 300, 1000, or 3000 SPZ/culture or medium alone. Cells were cultured for 5 days at 37°C in a humidified atmosphere containing 5% CO_2. During the final 16 h of culture, 1 μCi of [^{3}H]TdR (sp. act., 6.7 Ci/mM; DuPont NEN, Boston, MA) was added to each well to quantitate proliferative activity. Cultures were harvested with an automated sample harvester (Skatron, Sterling, VA) and analyzed by scintillation spectroscopy.

Lymphocyte cultures for the induction of lymphokines

Cells were prepared as for the proliferation assay with the following exceptions: 12 replicate wells containing splenic lymphocytes and SPZ, ranging from 0 to 3000 SPZ/well, were cultured in three replicate plates to be assayed on different days. Supernatants were harvested and pooled as previously described (34). The supernatants were stored in polypropylene tubes (Falcon, Oxnard, CA) at −20°C until assayed.

IL-2 assay

CTLL, an IL-2-dependent cell line (a gift from Dr. Carol Hickman, Center for Disease Control, Atlanta, GA), was used as described previously for the determination of IL-2 (34). Briefly, 4×10^3 cells/well were dispensed into 96-well microtiter plates (Costar) and 50 μl of 24-h culture supernatant were added in triplicate in twofold serial dilutions yielding final concentrations of 50%, 25%, 12.5%, 6.25%, and 3.125%. CTLL cells were incubated with rIL-2, ranging from 0 U to 10,000 U/ml (Genzyme, Cambridge, MA) to generate a standard curve. Cultures were incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO_2 and, during the last 16 h, cultures were pulsed with 1 μCi [^{3}H]TdR. Cell cultures were harvested and processed for scintillation spectroscopy. IL-2 levels are expressed as cpm.

IL-4 assay

CT4.R, an IL-2-dependent, IL-4-responsive cell line (a gift from Dr. William Paul, National Institutes of Health), was used as described previously (34). Briefly, 5×10^4 cells/well were cultured in 96-well microtiter plates (Costar) with serial twofold dilutions of 48-h culture supernatant ranging from 50% to 3.125%. As controls, cells were cultured in medium alone or with rIL-4 ranging from 0.001 to 10,000 U/ml. Cultures were incubated for 48 h at 37°C in a humidified atmosphere containing 5% CO_2 and, during the last 16 h, cultures were pulsed with 1 μCi [^{3}H]TdR. Cell cultures were harvested and processed for scintillation spectroscopy. IL-4 levels are expressed as cpm.

IFN-γ assay

WEHI 279.1.4, an IFN-γ-sensitive cell line (a gift from The Jackson Laboratory), was used for the determination of IFN-γ (34). Briefly, 5×10^4

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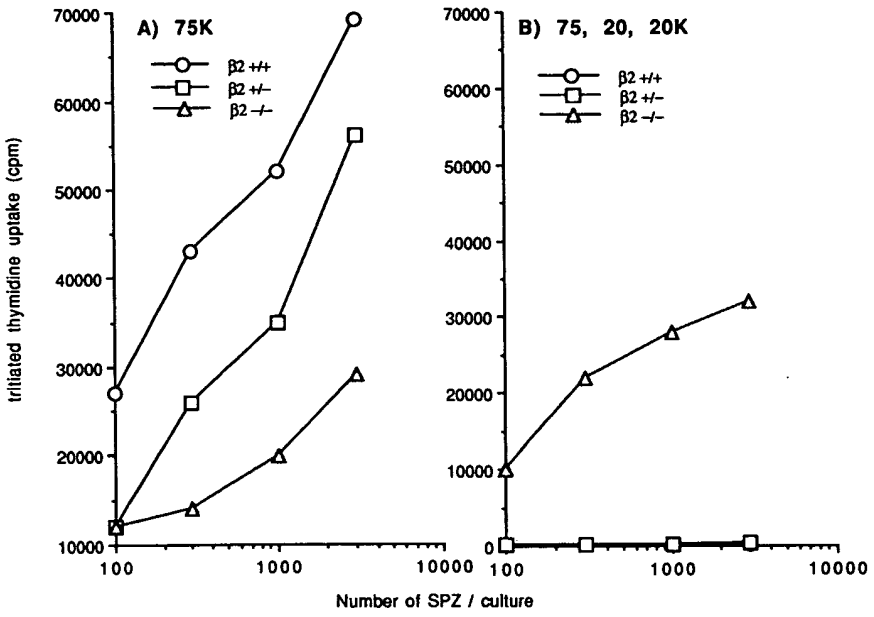

FIGURE 1. Anti-SPZ proliferative responses. Splenic lymphocytes (5×10^6/ml) from (*A*) SPZ-primed (75,000) and (*B*) SPZ-immune (75,000, 20,000, and 20,000) $\beta_2m^{+/+}$ (circles), $\beta_2m^{+/-}$ (squares), and $\beta_2m^{-/-}$ (triangles) mice were stimulated for 5 days with SPZ Ag ranging from 100 to 3000 SPZ per culture or with medium alone. Data from a representative experiment are expressed as Δ cpm of the mean of [^{3}H]TdR incorporation in triplicate wells. The cpm in the medium control cultures was <300. SEM < 10%.

cells/well were dispensed into triplicate wells containing twofold dilutions of 96-h culture supernatant ranging from 12 to 1.5%. WEHI 279.1.4 cells were cultured with medium alone or supplemented with rIFN-γ (Genzyme) at 1000 U/ml as a control. After 48-h incubation at 37°C in a humidified atmosphere containing 5% CO_2, cell cultures were pulsed with 1 μCi [^{3}H]TdR, harvested, and processed for scintillation spectroscopy.

Anti-CSP IgG determination

Serum anti-CSP IgG was determined from the tail vein blood of individual mice (34). Briefly, microtiter plates (96-well, U-bottom Immulon-2 polystyrene; Costar) were coated with 50 μl of *P. berghei* CSP octamer repeat $(DPAPPNAN)_3$ synthetic peptide in PBS (0.1 μg/50 ml) and allowed to incubate for 16 h at room temperature. Following a wash, the wells were blocked by addition of 300 μl casein buffer. After a 1-h incubation at room temperature, the casein buffer was aspirated and 50 μl of serum from individual mice in casein buffer at dilutions ranging from 1:50 to 1:6400 were added in triplicate and allowed to incubate for 2 h at room temperature. After six washes in PBS-TW20, 50 μl of a 1:500 dilution of goat anti-mouse–peroxidase IgG (Bio-Rad) was added to each well and incubated at room temperature for 1 h. The reaction was stopped by adding 10 μl of 20% SDS and the absorption measured at 415_{nm} with a Titertek Multiskan microplate reader. The results are expressed as the reciprocal of the serum dilution yielding a positive reading of 1.00 OD_{415} U.

Cytofluorometry

Splenic lymphocytes were analyzed for the cell surface expression of CD4, CD8, TCR-αβ, TCR-γδ, or NK1.1 by FACS. Briefly, 1×10^6 cells/ml were resuspended in PBS (supplemented with 0.5% FBS, 0.1% sodium azide) and labeled with FITC- or phycoerythrin (PE)-conjugated Ab to CD4, CD8, TCR-αβ, TCR-γδ, or NK1.1 (Boehringer Mannheim Corp., Indianapolis, IN) for 1 h on ice in the dark. Cells were washed three times in PBS and fixed with 1% formaldehyde. Cells were analyzed immediately on a FACScan (Becton Dickinson, Braintree, MA).

Results

We have previously established that immunization with attenuated *P. berghei* SPZ induces Ag-specific splenic proliferative T cell responses, IFN-γ, IL-2, anti-CSP-IgG, as well as protective immunity (33, 34). These responses are dependent on both the murine strain and the immunizing dose of irradiated SPZ (33). For example, BALB/c mice primed with 75,000 SPZ are fully protected against an infectious challenge, but the induction of proliferative T cell responses requires a boost immunization (33). Sporozoite-immune spleen cells from C3H/HeN mice are typically refractory to in vitro recall with SPZ Ag, unless the responding lymphocytes are depleted of $CD8^+$ T cells (33). Interestingly, expansion of $CD4^+$ T cells has always accompanied induction of protective immunity that requires several boost immunizations with irradiated SPZ (33). In contrast, SPZ priming of C57BL/6 mice induces proliferative splenic T cells and IFN-γ production to SPZ Ag in vitro, but two additional SPZ boost immunizations are required for induction of protective immunity, at which time the proliferative response declines to background levels but can be revealed after deletion of $CD8^+$ T cells (34).

Attenuated SPZ induce immune responses in $\beta_2m^{-/-}$ mice

In the first set of experiments, we characterized anti-SPZ proliferative reactivities of spleen cells from SPZ-primed $\beta_2m^{-/-}$ mice by comparing them to splenic responses elicited from wild-type, C57BL/6 ($\beta_2m^{+/+}$), and heterogeneous ($\beta_2m^{+/-}$) mice. Splenic cells from all three groups proliferated in vitro in a dose-related manner to SPZ Ags (Fig. 1*A*), although the splenic proliferative reactivity of $\beta_2m^{-/-}$ mice was lower than in the other two groups. As expected, splenic proliferative responses of $\beta_2m^{+/+}$ and $\beta_2m^{+/-}$ mice declined after the boost immunizations (Fig. 1*B*), whereas proliferative reactivity in $\beta_2m^{-/-}$ lymphocyte cultures increased (Fig. 1*B*). On the basis of this and previous observations (33), immunization with SPZ induces regulatory $CD8^+$ T cells in $\beta_2m^{+/+}$ but not in class I-deficient mice.

Because SPZ-induced protective immunity has been thought to be multifactorial, involving functionally and phenotypically diverse T cell sets (9–12) and Ab responses (13), we examined whether the absence of MHC class I molecules affected other responses elicited by attenuated SPZ. Cytofluorometric analysis of splenic T cells expressing CD4, TCR-αβ, and TCR-γδ showed that the numbers of these cells did not differ markedly between SPZ-immune $\beta_2m^{+/+}$, $\beta_2m^{+/-}$, and $\beta_2m^{-/-}$ mice (Table I). Somewhat reduced numbers of cells expressing the NK 1.1 marker were observed in $\beta_2m^{+/-}$ (15.3 to 16.4%) and $\beta_2m^{-/-}$ (12.5 to 28.2%) mice compared with $\beta_2m^{+/+}$ mice (31.1 to 35.2%) (Table I). The analysis was performed on more than 40 individual animals per strain and the data represent the range of percentages of the cell types measured for individual mice from each group. Occasionally, $CD8^+$ splenic T cells from a $\beta_2m^{-/-}$ animal were above the

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Table I. *SPZ-induced cell populations*[a]

Mouse Strain	% Positive Cells			
	CD4αβ	CD8αβ	γδ	NK
$\beta_2m^{+/+}$	9.0–13.7	12.8–17.2	1.7–7.8	31.1–35.2
$\beta_2m^{+/-}$	6.2–11.3	6.7–13.2	0.9–3.8	15.3–16.4
$\beta_2m^{-/-}$	6.2–13.3	0.6–5.7	1.7–8.0	12.5–28.2

[a] Splenic lymphocytes from SPZ-immunized animals were stained using FITC- or phycoerythrin-labeled mAb specific for CD4, CD8, TCR-αβ, TCR-γδ, or the NK marker. Data are represented as the range of percentages observed from 40 individual mice per group.

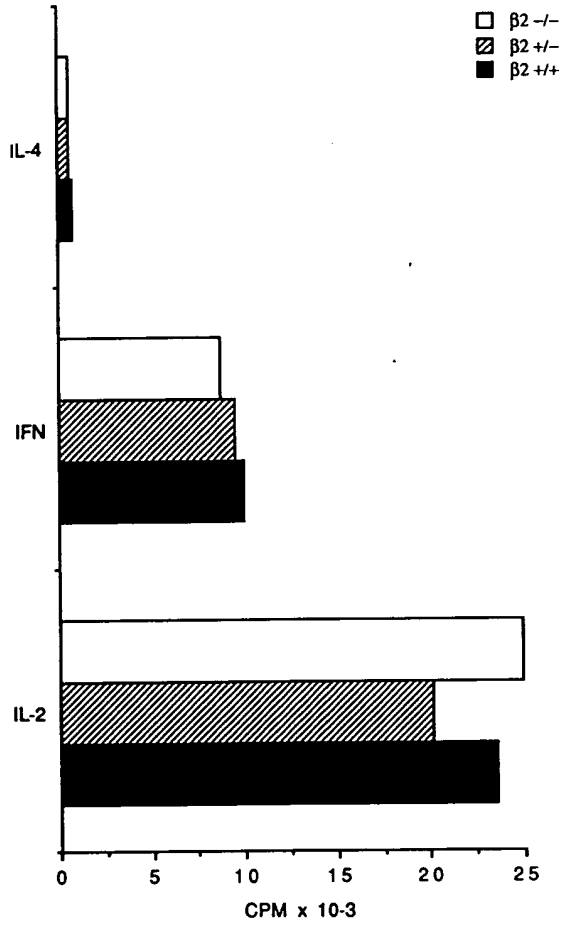

FIGURE 2. SPZ Ag induces Th1 cells in $\beta_2m^{+/+}$, $\beta_2m^{+/-}$, and $\beta_2m^{-/-}$ mice. Splenic cultures from $\beta_2m^{+/+}$, $\beta_2m^{+/-}$, and $\beta_2m^{-/-}$ mice primed with (75,000) irradiated SPZ were established with medium alone or with 1000 SPZ/ml. CTLL cells were used for the determination of IL-2 responses, CT4.R cells for IL-4, and WEHI 279.1.4 cells for IFN-γ. As controls, indicator cell lines were incubated with rIL-2, rIL-4, or rIFN-γ, or with medium alone for positive and negative responses as described in *Materials and Methods*. Data from representative experiments are expressed as Δ cpm of [^{3}H]TdR incorporation in triplicate wells. WEHI279.1.4 cells were IFN-γ sensitive and cpm for control cultures containing medium alone or 1000 U/ml rIFN-γ were 127,978 and 5285, respectively. SEM < 10%.

expected low levels (1 to 2%), however, these numbers never exceeded 5.7% of the total splenic lymphocytes (Table I).

Lymphokine analysis of SPZ-immune splenic lymphocytes revealed that IL-2 and IFN-γ responses were comparable between $\beta_2m^{+/+}$, $\beta_2m^{+/-}$, and $\beta_2m^{-/-}$ SPZ-stimulated cultures (Fig. 2), but, again, IL-4 was not induced (34) (Fig. 2). Elevated anti-CSP

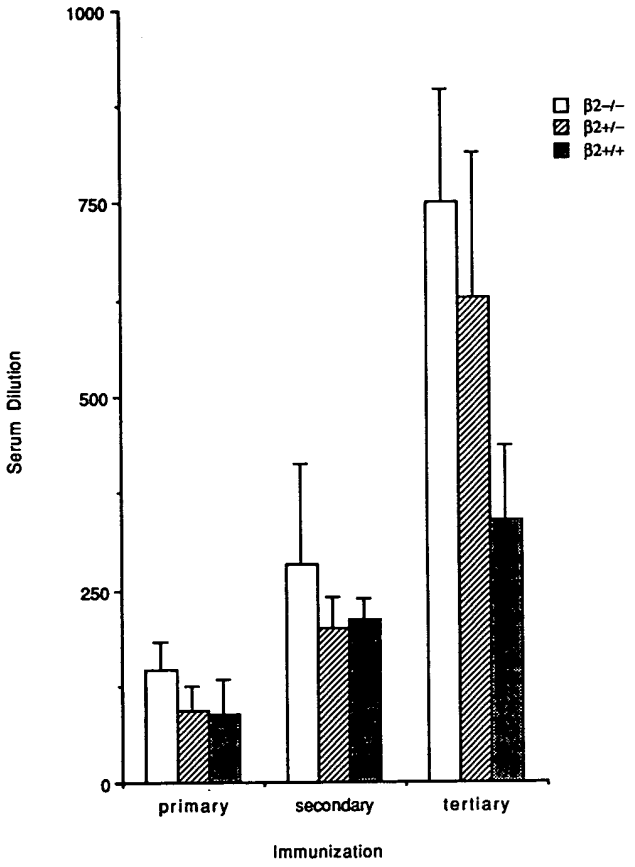

FIGURE 3. Anti-CSP Ab production. Ab titers were determined by ELISA from the serum following the primary, secondary, and tertiary immunizations in $\beta_2m^{+/+}$ (black bars), $\beta_2m^{+/-}$ (striped bars), and $\beta_2m^{-/-}$ (white bars) mice. Data are expressed as the reciprocal serum dilution yielding an OD_{415} of 1.00.

repeat specific IgG Ab responses were observed in all SPZ-immune groups ($\beta_2m^{+/+}$, $\beta_2m^{+/-}$, and $\beta_2m^{-/-}$), although levels fluctuated among individual mice and $\beta_2m^{-/-}$ mice showed the lowest responses (Fig. 3).

Attenuated SPZ fail to induce protection in $\beta_2m^{-/-}$ mice

In the next set of experiments, we sought to establish whether the absence of the MHC class I molecules in $\beta_2m^{-/-}$ mice would affect protective immunity induced by attenuated *P. berghei* SPZ. After SPZ priming and two boost immunizations, $\beta_2m^{+/+}$ and $\beta_2m^{+/-}$ mice were solidly protected against a 10,000 infectious *P. berghei* SPZ challenge (Fig. 4*A*). In contrast, identical immunization with attenuated SPZ failed to protect $\beta_2m^{-/-}$ mice, and all SPZ-immune $\beta_2m^{-/-}$ mice became parasitemic by day 9 post-challenge (Fig. 4*A*), as did the naive control wild-type mice (data not shown). The results were reproduced using more than 40 animals per group and different SPZ preparations.

Because different murine strains have dose-dependent requirements for the induction of protection as well as susceptibility to malaria infection (35), we investigated whether reducing the challenge dose from 10,000 to 1000 might reveal protective immunity. As shown in Figure 4*B*, limiting the infectious challenge dose also led to parasitemia in SPZ-immune $\beta_2m^{-/-}$ mice, whereas the SPZ-immune $\beta_2m^{+/+}$ mice were solidly protected. Considering the possibility that $\beta_2m^{-/-}$ mice might require a higher dose of

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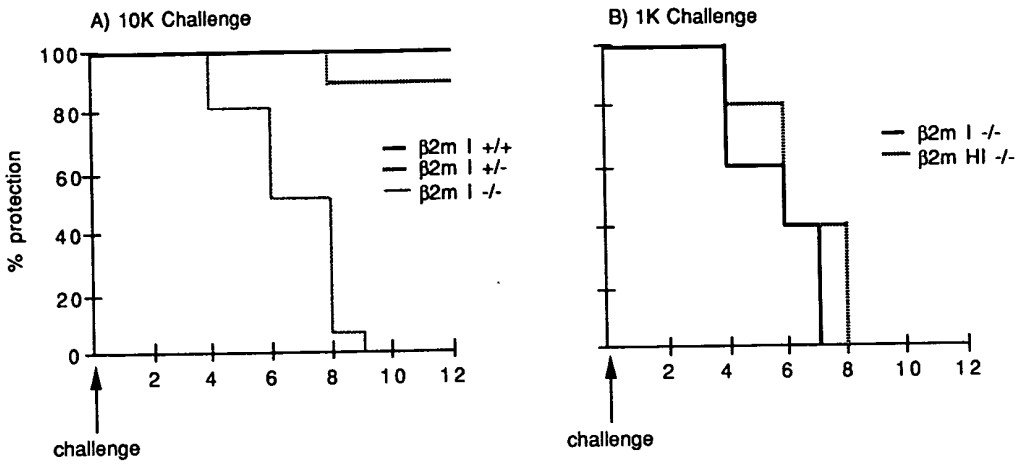

FIGURE 4. Irradiation attenuated *P. berghei* SPZ fail to induce protective immunity in $\beta_2m^{-/-}$ mice. Mice immunized with 75,000, 20,000, and 20,000 irradiated *P. berghei* SPZ were assumed immune (I) ($\beta_2m^{+/+}$ I, $\beta_2m^{+/-}$ I, and $\beta_2m^{-/-}$ I), and those immunized with 100,000, 20,000, 20,000, and 20,000, hyperimmune (HI) ($\beta_2m^{-/-}$ HI). One week after the final immunization, mice were challenged with either 10,000 (*A*) or 1,000 (*B*) infectious *P. berghei* SPZ. Data represent cumulative results from five separate experiments including more than 40 mice per group and are expressed as percentage of protection. $\beta_2m^{+/+}$ and $\beta_2m^{+/-}$ mice that were negative for parasites on day 12 (*A*) were still negative on day 25 (data not shown).

attenuated SPZ to induce protective immunity, we hyperimmunized with 100,000 followed by three weekly 20,000 boost immunizations, before a 1000 infectious SPZ challenge. Hyperimmunization also failed to protect $\beta_2m^{-/-}$ mice and all animals developed parasitemia within 8 days after the 1000 challenge (Fig. 4*B*). Thus, in contrast to $\beta_2m^{+/+}$ and $\beta_2m^{+/-}$ mice, $\beta_2m^{-/-}$ mice fail to develop protective immunity induced by immunization with attenuated SPZ.

Protective immunity is not transferable with SPZ-immune splenic cells

Because adoptive transfer of SPZ Ag-specific T cells confers protection against an infectious SPZ challenge (14, 24), we transferred SPZ-immune splenic cells from protected β_2m-competent donor mice to naive $\beta_2m^{-/-}$ recipients. We chose to transfer the entire splenic population in preference to enriched $CD8^+$ T cells to ensure the availability of the necessary inducer cells as well as a full complement of MHC class I molecules. According to the data presented in Figure 5*A*, transfers of 1×10^8 SPZ-immune splenic lymphocytes from $\beta_2m^{+/+}$ donors into naive $\beta_2m^{+/+}$ recipients resulted in 100% protection, whereas an identical transfer of non-immune donor splenic cells into naive recipients resulted in parasitemia (data not shown). Similarly, 80% protection was achieved with adoptively transferred SPZ-immune $\beta_2m^{+/+}$ splenic lymphocytes into naive $\beta_2m^{+/-}$ mice, and, conversely, with SPZ-immune $\beta_2m^{+/-}$ spleens into $\beta_2m^{+/+}$ naive recipients (Fig. 5*A*). In contrast, neither SPZ-immune $\beta_2m^{+/+}$ nor $\beta_2m^{+/-}$ splenic cells conferred protection upon transfer to naive $\beta_2m^{-/-}$ recipients (Fig. 5*B*). Pre-immunization of $\beta_2m^{-/-}$ recipients with irradiated SPZ followed by adoptive transfer of SPZ-immune spleen cells from $\beta_2m^{+/+}$ donors was equally ineffective in protecting the recipients against infectious SPZ challenge (Fig. 5*B*). Despite the lack of protection, Abs specific for CSP were detected in serum from the $\beta_2m^{-/-}$ recipients (data not shown), hence demonstrating that induction of a B cell response is insufficient for effective protection against an infective SPZ challenge.

Conversely, we examined whether SPZ-immune (75,000, 20,000, 20,000) $\beta_2m^{-/-}$ splenic cells might contain lymphocyte subsets capable of conferring protection to $\beta_2m^{+/+}$ or $\beta_2m^{+/-}$ naive recipients. Under these conditions, all adoptively transferred recipient mice succumbed to malaria infection (data not shown), confirming that SPZ immunization of $\beta_2m^{-/-}$ mice does not expand lymphocytes that engage in an effector function leading to protective immunity.

Discussion

T cell-mediated responses are believed to play a key role in modulating pathogenesis of the SPZ-stage infection. Although functionally diverse T cells (9–13) have been found to mediate anti-malaria effects, studies from murine and human models of protective immunity induced by irradiated SPZ have suggested that $CD8^+$ CTL are the primary effectors of anti-SPZ protective immunity (21, 23). Despite evidence for the critical role of $CD8^+$ CTL, neither the cellular nor the molecular requirements for $CD8^+$ T cell induction or for recognition of malaria Ags are known. The $\beta_2m^{-/-}$ animals provide an excellent model with which to investigate the in vivo processing and presentation of exoerythrocytic Ags in the absence of MHC class I molecules, and by extension, the cellular and molecular context for the involvement of $CD8^+$ T cells in protective immunity induced by attenuated SPZ.

MHC class I molecules are essential for protection induced by attenuated SPZ

The presence of class I molecules, particularly in the thymus, is critical for the development of a functional $CD8^+$ T cell repertoire and in the periphery MHC class I molecules restrict Ag-specific cytolytic responses of $CD8^+$ T cells (36). Typically, antigenic peptides derived mainly from cytosolic or nuclear proteins are transported into the endoplasmic reticulum where they bind to MHC class I heavy chain and β_2m, forming a trimolecular complex that is transported to the cell surface to activate $CD8^+$ T cells (37). Immunogenic peptides corresponding principally to viral Ags have been isolated from class I molecules expressed on virally infected cells and have been shown to activate specifically $CD8^+$ T cells

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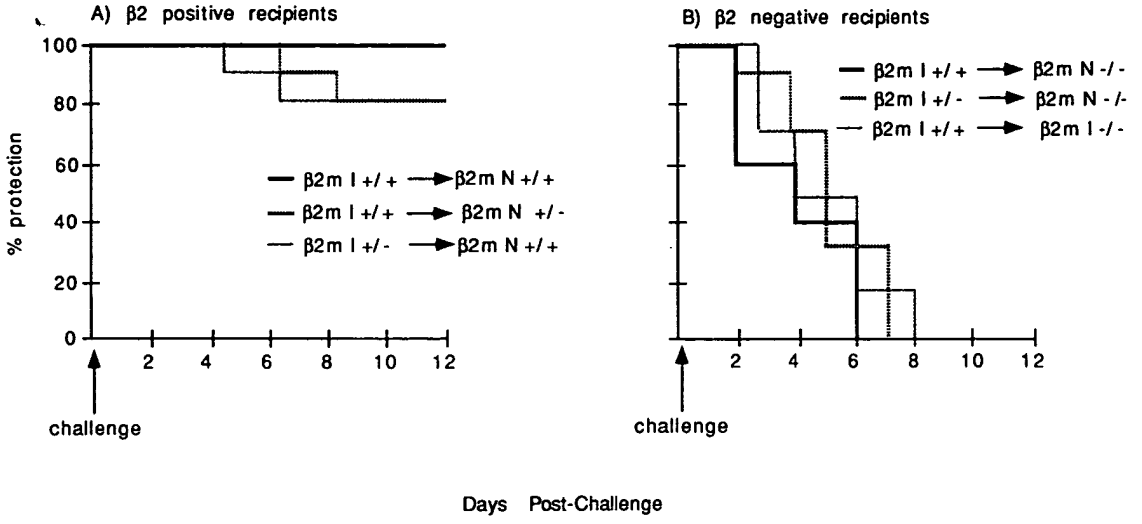

FIGURE 5. Adoptive transfer of SPZ-immune splenic cells fails to confer protection from $\beta_2m^{+/+}$ to $\beta_2m^{-/-}$ mice. Naive (N) or immune (I) mice, immunized with 75,000, 20,000, and 20,000 irradiated *P. berghei* SPZ, were used as donors or recipients of entire splenic cell population transfers (1×10^8 cells per mouse transferred i.v.). In *A*, β_2m^+ recipients were used; in *B*, β_2m^- recipients were used. Data represent results from five separate experiments including more than 40 mice per group and are expressed as percentage of protection. $\beta_2m^{+/+}$ and $\beta_2m^{+/-}$ mice that tested negative for parasites on day 12 (*A*) were still healthy on day 25 (data not shown).

(38). Peptides derived from tumor and microbial Ags (38) have also been found to be associated with class I molecules for presentation to $CD8^+$ T cells. Such information, however, is missing for malaria Ags under conditions of natural infection and attenuated SPZ immunization. Nonetheless, CTLs specific for peptides corresponding to exoerythrocytic Ags have been detected in malaria-immune mice (39) and humans (20).

The observations presented here unequivocally establish that MHC class I molecules are essential for SPZ-induced protective immunity, because mice with the disrupted β_2m gene cannot be protected. The failure to generate protective immunity was demonstrated by the inability of large doses of irradiated SPZ, and the ineffectiveness of adoptively transferred SPZ-immune splenic cells, to protect against an infectious challenge. Assuming that irradiated SPZ colonized the liver and developed into liver-stage parasites, the lack of class I expression on hepatocytes or on APCs in the liver prohibited the processing and presentation of liver-stage Ags. Consequently, $CD8^+$ T cells were not induced during immunization with attenuated SPZ. Furthermore, in the adoptive transfer experiments, $CD8^+$ T cells from SPZ-immune $\beta_2m^{+/+}$ donors were not properly engaged by $\beta_2m^{-/-}$ hepatocytes to function as antimalaria effector cells. Our results, therefore, support the view that class I molecules are involved in transport and presentation of peptides derived from liver-accumulated Ags for activation of $CD8^+$ T cells, a process that is clearly missing in $\beta_2m^{-/-}$ animals. In the absence of direct evidence regarding the site and mechanism by which attenuated SPZ-derived Ags are processed and presented, it can be speculated that infected hepatocytes, Kupffer or liver-resident dendritic cells, present processed malaria Ags expelled from the infected liver cells to class I-restricted $CD8^+$ T cells.

Although it has been shown that a subset of D^b molecules can be exported to the cell surface without β_2m (40), it is clear from this study that either malaria Ags do not associate with the H-$2D^b$ class I molecules or suboptimal concentrations of H-$2D^b$ prohibit activation of effector cells in $\beta_2m^{-/-}$ mice. It has been demonstrated that MHC class I-like CD1 molecules associate with β_2m and activate murine $NK^+TCR\text{-}\alpha\beta^+$ cells and a subpopulation of $CD4^+$ T cells (41, 42). However, it remains unknown whether these cells participate in malaria-specific protective immunity.

Local expression of class I molecules on the target cells is critical for induction of $CD8^+$ T cells and protective immunity

To overcome the inability of the $\beta_2m^{-/-}$ mice to generate protective immunity by immunization with irradiated SPZ, we adoptively transferred SPZ-immune wild-type splenic cells containing SPZ-specific $CD8^+$ effector T cells and a full complement of accessory cells. We presumed that a portion of the MHC class I molecules on the transferred donor cells would be occupied by peptides derived from processed attenuated SPZ Ags and that such occupancy might engage the transferred SPZ-immune $CD8^+$ T cells in the antiparasitemic immune mechanism. The failure of the immunologically competent SPZ-immune splenic cells to prevent parasitemia in the $\beta_2m^{-/-}$ recipients presumably resulted from the inability of $\beta_2m^{-/-}$ infected liver cells to engage donor cells owing to the lack of class I molecules on the $\beta_2m^{-/-}$ hepatocytes. Induction and expansion of cytolytic peritoneal $CD8^+$ T cells has been elicited in $\beta_2m^{-/-}$ mice by immunization with the tumor cell lines P815 and EL-4, because the MHC class I molecules on these tumor cells provided the necessary activation signal through the TCR of the $CD8^+$ T cells (27).

It can be suggested that the ineffectiveness of the adoptively transferred $CD8^+$ T cells might have resulted from their failure to home to the liver, which is the site where anti-SPZ effects have been seen (39, 43–45). However, the success of this approach in the $\beta_2m^{+/+}$ recipients argues against this possibility, unless cellular tropism is somehow linked to the expression of class I molecules. Hence, our data suggest the need for MHC class I molecules during both the inductive and the effector phase of protective immunity.

Our observation then excludes the possibility that elimination or reduction of the parasite is accomplished by nonspecific mechanisms, including a host of lymphokines, and implies that the targets for effector cells are nonsplenic and of host origin. Recently, a study detailing *Plasmodium yoelii* protective immunity in the

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IFN-γ receptor knockout mice demonstrated that the order of Ag-specific cell activation is critical for the induction of protection (43) and our data fully support this conclusion.

Lack of compensatory mechanism in protective immunity

It has been demonstrated that in the absence of αβ T cells, partial protection to blood-stage infection with *P. yoelii* (46) and *P. adami* (47) is provided by the residual γδ T cells. Compensatory mechanisms involving MHC class II-restricted $CD4^+$ CTL have also been observed in virally induced pathologies, in which the eradication of viral infections seemed to be dependent exclusively on the class I-restricted $CD8^+$ T cells (28, 29). In *Toxoplasma gondii* infection, the NK activity was observed as a mechanism that remained fully functional, conferring protection on $\beta_2m^{-/-}$ mice (31), although suppressed NK activity has been reported in $\beta_2m^{-/-}$ mice (48). In contrast, no compensatory mechanisms were observed in the irradiated SPZ-immune $\beta_2m^{-/-}$ mice. In fact, a small reduction of NK cells from $\beta_2m^{-/-}$ mice was observed compared with $\beta_2m^{+/+}$ animals (Table I). $CD8^+$ T cells might be a crucial source of lymphokines for the expansion of liver-resident NK cells, and we are currently investigating the contribution of these cells in the model of protective immunity induced by attenuated SPZ.

It has been shown that under the influence of elevated IL-2 titers, $CD4^-/CD8^-$ T cells can be converted into single-positive T cells (49). Although SPZ immunization induced proliferative $CD4^+$ T cells, secreting IL-2 and IFN-γ, $CD8^+$ T cells were neither recruited nor activated in SPZ-immune $\beta_2m^{-/-}$ mice. Hence, specific activation of $CD8^+$ CTL by malaria Ags presented by MHC class I is required, rather than nonspecific recruitment by lymphokines or other cellular influences.

The role of $CD8^+$ T cells in protection against blood-stage malaria has been investigated in the β_2m knockout mouse model and data from this study suggests that clearance of blood-stage malaria was independent of $CD8^+$ T cells (50). Alternatively, $CD8^+$ T cells could be involved in the resolution of blood-stage malaria, but unlike in our study, compensatory mechanisms were activated in $\beta_2m^{-/-}$ mice infected with blood-stage parasites.

In summary, these data establish that in the absence of MHC class I molecules, protective immunity cannot be induced by attenuated SPZ and SPZ-immune T cells cannot transfer protective immunity, suggesting a key functional role for the MHC class I molecules and restricted $CD8^+$ T cells in the regulation of SPZ-induced pathogenesis. Although this might represent the main mechanism of protection only in C57BL/6 mice, several previously published studies in other murine strains have also demonstrated the need for $CD8^+$ T cells in imparting protective immunity (22–24). Our study not only corroborates these observations, but, most importantly, demonstrates the immutable link between MHC class I molecules and $CD8^+$ T cells, which is maintained in vivo during responses to irradiated SPZ. The importance of the MHC class I molecules was shown not only during the induction of the protective mechanism, but more strikingly during the effector phase. The presence of class I molecules on the adoptively transferred $\beta_2m^{+/+}$ splenic cells was completely ineffective in rendering protection, suggesting that the expression of the class I Ags in the liver, which is presumed to be the target for the effector mechanism, is required for the proper function of SPZ-immune spleen cells involved in protection.

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